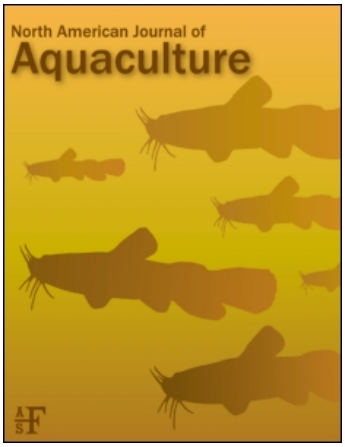

North American Journal of Aquaculture
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t926380648

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Emily R. Cornwell[a]; Geoffrey H. Groocock[a]; Rodman G. Getchell[a]; Paul R. Bowser[a]
[a] Aquatic Animal Health Program, Department of Microbiology and Immunology, College of Veterinary Medicine, Cornell University, Ithaca, New York, USA

First published on: 02 February 2011

To cite this Article Cornwell, Emily R. , Groocock, Geoffrey H. , Getchell, Rodman G. and Bowser, Paul R.(2011) 'Residual Tannic Acid Destroys Virucidal Properties of Iodophor', North American Journal of Aquaculture, 73: 1, 8 — 12, First published on: 02 February 2011 (iFirst)
To link to this Article: DOI: 10.1080/15222055.2011.544623
URL: http://dx.doi.org/10.1080/15222055.2011.544623

North American Journal of Aquaculture 73:8–12, 2011

ISSN: 1522-2055 print / 1548-8454 online
DOI: 10.1080/15222055.2011.544623

ARTICLE

Residual Tannic Acid Destroys Virucidal Properties of Iodophor

Emily R. Cornwell,* Geoffrey H. Groocock, Rodman G. Getchell, and Paul R. Bowser

Aquatic Animal Health Program, Department of Microbiology and Immunology, College of Veterinary Medicine, Cornell University, Ithaca, New York 14853, USA

Abstract

Surface de-adhesion and disinfection of eggs at hatcheries are critical steps in successful fry culture; however, little is known about the effects of the combination of these compounds on the efficacy of egg surface disinfection. We examined the effect of tannic acid on detection of viral hemorrhagic septicemia virus (VHSV) type IVb alone and in the presence of iodophor under in vitro conditions. We confirmed that tannic acid inhibits the quantitative reverse-transcriptase polymerase chain reaction assay developed in our laboratory and that it binds to VHSV, reversibly decreasing infectivity. Surprisingly, we also found that when combined with iodophor, tannic acid destroys the ability of either compound to inhibit infectivity. While we still strongly encourage the use of iodophor for its antiviral, bactericidal, and fungicidal properties, our results suggest that when tannic acid is used prior to disinfection with iodophor, it must be very thoroughly rinsed to ensure that it does not interfere with the disinfectant properties of iodophor.

Surface disinfection of eggs in a hatchery setting is crucial to limiting the spread of disease within the hatchery as well as in the bodies of water that will receive the hatched fry. With the recent introduction of viral hemorrhagic septicemia virus (VHSV) into the Great Lakes watershed (Elsayed et al. 2006; Groocock et al. 2007; Lumsden et al. 2007), proper disinfection has become even more important. Many fish viruses, including VHSV, can be found in ovarian fluids and as external contaminants of eggs (Jørgensen 1970; Amos et al. 1998). In fact, the stability of VHSV is increased in the presence of ovarian fluids (Kocan et al. 2001).

Early disinfection methods included the use of sulfomerthiolate and acriflavine, but their use was discontinued when they were shown to be bacteriostatic rather than bactericidal (McFadden 1969). Amend and Pietsch (1972) tested the virucidal effects of several additional compounds, including malachite green, thimerosal, sodium chloride, sodium hypochlorite, benzethonium chloride, formalin, and iodophor against the infectious hematopoietic necrosis virus, infectious pancreatic necrosis virus, and VHSV. Of all the tested disinfectants, only formalin, chlorine, and iodophor were effective, and iodophor disinfection gave the most consistent deactivation of the virus. In addition, iodophor is an effective disinfectant over a wide range of water hardness and pH (Amend and Pietsch 1972) and is effective against VHSV in both salt water and freshwater (Kurita et al. 2002). A recent thorough testing of the bactericidal and virucidal properties of iodophor provided standards for determining the effectiveness of disinfectants used in aquaculture and confirmed the efficacy of several iodophor-based products (Verner-Jeffreys et al. 2009).

Presently, iodophor is a commonly used egg disinfectant because it has antibacterial (Cipriano et al. 2001), antifungal (Khodabandeh and Abtahi 2006), and antiviral (Bullock et al. 1976; Drennan et al. 2006) properties and does not appear to adversely affect fry survival (Bouchard and Aloisi 2002; Dabrowski et al. 2009). Additionally, a recently developed protocol for iodophor-based surface disinfection of eggs takes advantage of egg development. Significant absorption of water

*Corresponding author: erc58@cornell.edu
Received February 14, 2010; accepted May 27, 2010
Published online February 2, 2011

occurs during the first 30 min after fertilization, theoretically allowing for absorption of iodophor and internal egg disinfection (Leary and Peterson 1990; Fowler and Banks 1991; Erdahl 1994; Bouchard and Aloisi 2002). This procedure involves placing eggs in a 50-mg/L iodophor solution for 30 min immediately after fertilization.

The eggs of many fish species have a jelly layer that must be removed or rendered nonadherent to allow the free movement of eggs in hatching jars and to avoid fungal infections in the jars (Bouchard and Aloisi 2002). The two most common compounds used to render eggs nonadherent are tannic acid and Fuller's earth. However, the use of tannic acid is more advantageous as it reduces the egg processing time required, allowing the iodophor to come into contact with the egg earlier in the water-hardening process and increasing the chance that some internal disinfection may occur. Thus, in most hatcheries in New York State, tannic acid is used to remove the jelly layer on adherent eggs.

The effects of concurrent use of tannic acid and iodophor have been examined only recently. Bouchard and Aloisi (2002) showed that using iodophor in combination with tannic acid did not appear to adversely affect fry survival. Although eggs treated with tannic acid were smaller in diameter than those treated with Fuller's earth, Bouchard and Aloisi (2002) stated that this was probably due to the lack of a jelly layer in the tannic acid-treated eggs. While concurrent use of these compounds does not appear to affect the survival of eggs, the effectiveness of disinfection when using both compounds has not been established.

This study investigated the effects of tannic acid on the effectiveness of iodophor as an antiviral compound to determine whether iodophor disinfection used in combination with tannic acid treatment is an effective hatchery protocol to prevent the spread of egg-associated VHSV.

METHODS

For all experiments, sterile, filtered, dechlorinated municipal water (pH = 7.41; hardness = 120 mg/L) was used to prepare solutions of tannic acid (Sigma-Aldrich, St. Louis, Missouri) and iodophor (povidone iodine; Agilent, Santa Clara, California) as this source most closely resembled the conditions found in most hatcheries. Except where indicated otherwise, all solutions of tannic acid were made to 400 mg/L and all solutions of iodophor were made to 50 mg/L. These concentrations were chosen because they are the current standards used by walleye *Sander vitreus* hatcheries in New York State.

Effect of tannic acid and iodophor on viability of virus.—Eight test solutions were made in 50-mL conical tubes (BD Biosciences, San Jose, California; Table 1) containing tannic acid, iodophor, both, or neither. All solutions were stored at 15°C prior to inoculation with virus and for the duration of the experiment. The four control solutions contained 50 mL of viral storage medium composed of minimal essential medium with Hanks' salts (Gibco, Invitrogen, Carlsbad, California) prepared with 10% fetal bovine serum (Gibco), penicillin (200 international units/mL), streptomycin (200 μg/mL), glutamine (0.584 mg/mL; Gibco), and HEPES buffer (1 M, 0.015 mL/mL; Gibco); hereafter, this storage medium is referred to as HMEM-10. The four experimental solutions were inoculated with VHSV type IVb at 1.5×10^7 plaque-forming units (PFU)/mL. The isolate used in this experiment was the Michigan 2003 isolate (Elsayed et al. 2006). The viral titer was confirmed with a plaque assay as described by Plumb and Bowser (1983), with the following modifications: drained epithelioma papulosum cyprini (EPC) cell culture monolayers (American Type Culture Collection CRL-2872; Fijan et al. 1983; Winton et al. 2010) were inoculated with 0.5-mL serial viral dilutions in triplicate on six-well plates (Corning, Lowell, Massachusetts), incubated at 15°C for 1 h, and then overlain with 0.6125% methylcellulose (Fluka Biochemika, Milwaukee, Wisconsin) in HMEM-10 and incubated in sealed containers at 15°C.

Two 300-μL aliquots from each treatment were collected with a sterile pipette at the following time intervals: 0, 0.5, 1, 3, 6, 12, 24, and 72 h postexposure. One aliquot was immediately frozen at −80°C for quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) testing at the conclusion of the experiment, while the second was immediately placed on fresh monolayers of EPC cells.

The EPC cell monolayers grown in HMEM-10 were inoculated with 100 μL of sample in triplicate on a 48-well plate (Corning) as described in the American Fisheries Society Blue Book (AFS-FHS 2007). Cell monolayers were checked for cytopathic effect (CPE) daily and confirmed with at least one subsequent passage. All wells that failed to show CPE after 2 weeks were subjected to a blind passage and were monitored daily for two more weeks. Cytopathic effect was recorded as present or absent.

Viral RNA was isolated using a Qiagen RNeasy Mini Kit (Qiagen, Inc., Valencia, California) according to the manufacturer's directions. Briefly, stored aliquots of each treatment were thawed, combined with 300 μL of lysis buffer, and vortexed; RNA was then precipitated with 600 μL of 70% ethanol, washed according to the manufacturer's directions, and eluted in 50 μL of ribonuclease-free water. The RNA samples were stored at −20°C for no longer than 1 week before use in qRT-PCR.

The qRT-PCR method used was slightly modified from that described by Hope et al. (2010). Briefly, samples, a no-template control, and at least three standards were loaded onto a 96-well plate in triplicate. Each reaction contained 1× TaqMan One-Step Universal PCR Master Mix with No AmpErase (Applied Biosystems, Inc. [ABI], Foster City, California), 1× Multiscribe, 200 nM of forward primer, 200 nM of reverse primer, 200 nM of probe, and 5 μL of total RNA. The assay was performed on an ABI Prism Model 7500 sequence detector under the following conditions: 30 min at 48°C for reverse transcription, 10 min at 95°C for Ampli*Taq* activation, and 45 cycles of 15 s each at 95°C, followed by 1 min at 60°C for denaturing, annealing, and extension. Copy numbers in unknown samples were determined by calculation of a linear regression

TABLE 1. Effect of tannic acid (400 mg/L) and iodophor (50 mg/L) on detection of viral hemorrhagic septicemia virus (VHSV) type IVb (isolate MI03) in water by cell culture and quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR). Results are shown as mean (SD in parentheses) for all time points combined ($n = 8$). Each time point was run on qRT-PCR and cell culture in triplicate (CPE = cytopathic effects; ND = none detected).

Treatment	CPE detected?	Viral genome copies detected before dialysis	Viral genome copies detected after dialysis	Viral genome copies detected in postdialysis samples after iodophor treatment
Water alone	No	ND	ND	ND
Tannic acid	No	ND	ND	ND
Iodophor	No	ND	ND	ND
Tannic acid + iodophor	No	ND	ND	ND
Water + VHSV	Yes	4.94×10^7 (5.41×10^6)	7.35×10^2 (4.63×10^2)	3.05×10^3 (1.92×10^2)
Tannic acid + VHSV	Yes	ND	ND	1.18×10^3 (1.03×10^2)
Iodophor + VHSV	No	5.18×10^5 (3.09×10^4)		
Tannic acid + iodophor + VHSV	Yes	8.31×10^5 (8.83×10^4)		

from the standards. The VHSV primers used were (1) forward primer 5′-ACCTCATGGACATCGTCAAGG-3′; (2) reverse primer 5′-CTCCCCAAGCTTCTTGGGTGA-3′; and (3) probe 5′-/56-FAM/CCCTGATGACGTGTTCCCTTCTGACC/36-TAMSp/-3′.

Dialysis of tannic acid.—Current evidence shows that tannic acid inhibits the enzymatic reactions required for PCR (Kreader 1996), and previous preliminary experiments using tannic acid in our laboratory yielded ambiguous results. Therefore, the tannic acid solutions were dialyzed for 24 h by following a protocol similar to the one used by Verner-Jeffreys et al. (2009) with an acidic iodophor, except that dialysis tubing of 6,000–8,000 daltons (molecular-weight cut off) was used and dialysis was carried out at 15°C for 24 h in 2.0 or 3.5 L of water with 100% water changes at 3, 5, and 15 h. The RNA from solutions before and after dialysis was extracted and assayed using qRT-PCR as described above. To determine whether residual tannic acid from the dialysis was still interfering with complementary DNA synthesis or PCR, all samples containing tannic acid were loaded alone and loaded in the presence of 7.5×10^4 viral copies of standard.

Tannic acid neutralization.—A solution of 400-mg/L tannic acid was prepared using dechlorinated municipal water, which was sterile-filtered through a 0.2-μm membrane (0.4 g of tannic acid in 1 L of tap water). This solution was allowed to equilibrate to 15°C for 1 h. After equilibration, serial dilutions of tannic acid were prepared as summarized in Table 2. An aliquot of VHSV type IVb, which was previously shown to have a titer of 1.5×10^9 PFU/mL, was added to each dilution to a final concentration of approximately 1.5×10^7 PFU/mL. All samples were incubated at 15°C for 5 min to simulate hatchery conditions after addition of virus. An aliquot of iodophor was then added to each dilution for a final iodophor concentration of 50 mg/L; the mixtures were vortexed, and solutions were incubated at 15°C for 1 h to simulate hatchery conditions. At the end of 1 h, a 200-μL aliquot was used for RNA extraction and assayed using qRT-PCR, and 250 μL were immediately plated in triplicate on EPC cells (1:2 final dilution in well) as previously described.

RESULTS

There were significant differences in the detection of VHSV exposed to iodophor by cell culture and qRT-PCR at all time points, confirming that 50 mg/L iodophor is sufficient to destroy virus infectivity but not viral RNA. Even immediately after iodophor addition, no infectious virus was detected (Table 1). The results for tannic acid were less conclusive since some CPE was observed initially in cell culture for both the control and VHSV-infected solutions. However, the CPE was inconsistent on subsequent passage, and qRT-PCR assay of cell culture homogenates as well as original solutions failed to detect any virus (Table 1). Attempts to dialyze the tannic acid to avoid toxicity in cell culture were ineffective: no virus was detected in any tannic acid-treated samples before or after dialysis, even when extracted RNA from samples was spiked with a VHSV standard. However, when tannic acid and iodophor were added simultaneously, VHSV was detected in cell culture and isolated by PCR at all time points (Table 1). Titration of tannic acid in the presence of 50 mg/L iodophor demonstrated that a 1:100 or greater dilution of tannic acid (to a final concentration of 4 mg/L or less) is required to maintain the virucidal properties of iodophor for VHSV under these conditions (Table 2).

DISCUSSION

Exposure to 50-mg/L iodophor immediately destroyed the infectivity of VHSV, confirming the potent virucidal properties of iodophor. However, viral RNA was still detected for up to 72 h postexposure using qRT-PCR. This is probably due to the persistence of RNA in the environment (Rodríguez et al. 2009) and emphasizes the importance of confirming viral infectivity with cell culture.

TABLE 2. Summary of tannic acid dilutions used for the neutralization experiment ($n = 3$). Viral hemorrhagic septicemia virus (VHSV) genome copies are shown as mean with SD in parentheses (PFU = plaque-forming units; CPE = cytopathic effects; ND = none detected).

Tannic acid (mg/L)	Iodophor (mg/L)	VHSV (PFU/mL)	CPE detected?	Average number of viral genome copies detected
400	50	1.5×10^7	Yes	ND
40	50	1.5×10^7	Yes	2.45×10^5 (4.35×10^4)
4	50	1.5×10^7	No	8.31×10^5 (8.83×10^4)
0.4	50	1.5×10^7	No	5.18×10^5 (3.09×10^4)
0.04	50	1.5×10^7	No	1.84×10^5 (2.91×10^4)
0.004	50	1.5×10^7	No	2.90×10^5 (3.04×10^4)
0.0004	50	1.5×10^7	No	3.22×10^5 (1.16×10^4)
0.00004	50	1.5×10^7	No	2.71×10^5 (2.00×10^4)
400	0	1.5×10^7	Yes	ND
400	0	0	Yes	ND
0	50	0	No	ND
0	0	1.5×10^7	Yes	5.00×10^6 (4.20×10^4)

Tannic acid appears to reversibly inactivate VHSV. Virus incubated with tannic acid was undetectable using both PCR and cell culture, but addition of iodophor either at or after virus addition restored detection through both techniques. Tannic acid has been shown to reversibly inactivate other animal viruses as well, including the Chikungunya virus (an alphavirus; Konishi and Hotta 1979), influenza virus (Carson and Frisch 1953), Newcastle disease (Kucera and Hermann 1967), herpes simplex virus (Takechi et al. 1985), and poliovirus (Quignon et al. 1998). In particular, inactivation of Chikungunya virus by tannic acid is pH- and protein-dependent, which suggests that phenolic hydroxyl groups on tannic acid interact with proteins on the surface of the virus to inhibit infectivity (Konishi and Hotta 1979, 1980; Chung et al. 1998).

In addition to its role in virus inactivation, tannic acid is also a known inhibitor of nucleic acid and protein synthesis (Badawy et al. 1969). The polyphenolic structure of tannic acid binds RNA during extraction, rendering it inaccessible to reverse transcription and PCR enzymes (Tattersall et al. 2005). Tannic acid that remains associated with extracted RNA inhibits the activity of the enzymes used in both reverse transcription and PCR (Kreader 1996). This inhibitory effect can be reduced by the addition of a protein such as bovine serum albumin or the addition of another compound that interacts with the phenolic hydroxyl groups on tannic acid. Additionally, in our work, we found that iodophor negates the inhibitory effect of tannic acid.

Although it is clear that the presence of tannic acid destroys the disinfectant properties of iodophor under in vitro conditions, the exact interaction between tannic acid and iodophor remains relatively unknown. The normal action of iodophor is to allow the slow release of the free iodide ion, I^-, from the organic molecules. Once free in the aqueous solution, nine different equilibria can occur to form 10 different iodine species (Gottardi 1999). Two of these species, hypoiodus acid and elemental iodine, are responsible for destroying microbes and viruses through oxidation or halogenation (USFWS 2004). It appears likely that the tannic acid also is oxidized by iodophor, leaving few active molecules to disinfect egg surfaces.

Regardless of the precise mechanism, we have shown that a dilution of at least 1:100 of tannic acid is required to maintain the virucidal properties of iodophor under the conditions of this experiment. However, the experiment measured free virus in water without the presence of other proteins or organic material. We are currently testing the interaction between tannic acid and iodophor during fish egg disinfection to ensure that the egg surface itself does not confer protection to either viruses or tannic acid.

In this study we initially obtained some false positive results in cell culture because of the toxicity of tannic acid on EPC cells and some false negative results in qRT-PCR because of the inhibitory effect of tannic acid on PCR enzymes. Employing two separate methods allowed us to detect this interaction.

The effects of tannic acid on the virucidal properties of iodophor are of great concern for future effective management and disinfection of fish eggs. Hatcheries and researchers using tannic acid as an anti-clumping agent and iodophor as a disinfectant should continue to ensure that eggs are thoroughly rinsed between exposures to each compound. Our results suggest that a 1:100 dilution of tannic acid is sufficient to retain the virucidal properties of iodophor in an in vitro system, but this is likely to be dependent on the pH and presence of other organic compounds in the water.

ACKNOWLEDGMENTS

We thank Gregory Wooster (College of Veterinary Medicine, Cornell University) and Steven LaPan (New York State Department of Environmental Conservation) for technical support provided for this project. This publication is a resulting product from Project R/ATD-11 funded under Award NA07OAR4170010 from the National Sea Grant College Program of the U.S. Department of Commerce's National Oceanic and Atmospheric Administration to the Research

Foundation of the State University of New York on behalf of New York Sea Grant. The statements, findings, conclusions, views, and recommendations are those of the authors and do not necessarily reflect the views of any of these organizations.

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